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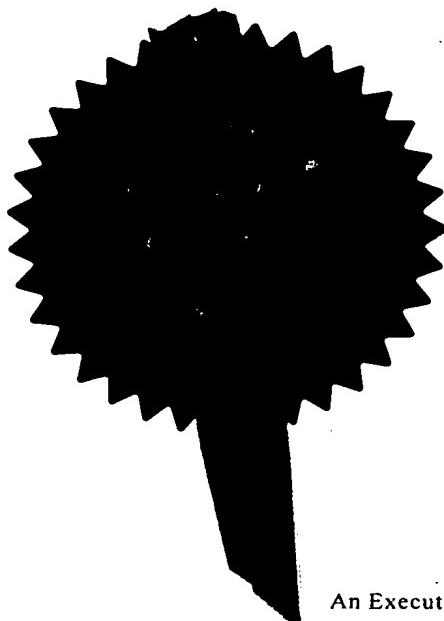
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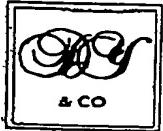


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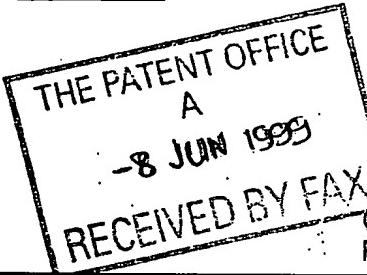
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The Patent Office

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1. Your reference

P006825GB CTH

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2. Patent application number  
(The Patent Office will fill in this part)3. Full name, address and postcode of the or of each applicant  
(underline all surnames)

K C L Enterprises Limited  
King's College London  
Waterloo Bridge House  
57 Waterloo Road  
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SE1 8WA  
United Kingdom

Patents ADP number (if you know it)

If the applicant is a corporate body, give the country/state of its incorporation

7674799001

Dy

4. Title of the invention

Product

5. Name of your agent (if you have one)

D YOUNG &amp; CO

"Address for service" in the United Kingdom to which all correspondence should be sent  
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Description 31

Claims(s) 2

Abstract 1

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Statement of inventorship and right to grant of a patent (Patent Form 7/77)

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Request for substantive examination (Patent Form 10/77)

Any other documents NO  
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Signature

Date

D. Young & Co

D YOUNG & CO  
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11. Name and daytime telephone number of the person to contact in the United Kingdom

Dr C T Harding

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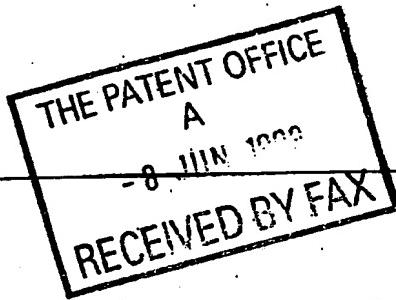
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PRODUCT

The present invention relates to a novel product. In particular, the invention relates to a novel product which has a broad applicability. More in particular, the invention relates to a novel product and its use as an industrial component such as an antifoulant, 5 or an anti-adhesive, or as a pharmaceutical component such as an anti-inflammatory agent.

Biofouling - the coating of surfaces by organic molecules or organisms - is a major problem, especially in damp or aquatic environments. Anti-fouling agents are required 10 for many different applications, particularly for marine structures which are exposed to sea water flora and fauna. Mildew or fungus may grow on house paints and the like, utilizing the paint medium as a nutrient, or in some cases using the underlying substrate, such as wood, as the nutrient. This may cause damage to the painted surface or a deterioration in the appearance of the painted surface. Slime or algae may develop 15 in water cooling towers if effective compounds for preventing their growth are not present. Anti-fouling agents are useful in combating these problems.

As discussed in US-A-5071479 the growth of marine organisms on the submerged parts of a ship's hull is a particular problem. Such growth increases the frictional 20 resistance of the hull to passage through water, leading to increased fuel consumption and/or a reduction in the speed of the ship. Marine growths accumulate so rapidly that the remedy of cleaning and repainting as required in dry-dock is generally considered too expensive. An alternative is to limit the extent of fouling by applying to the hull a 25 paint incorporating anti-fouling agents. These prior art anti-fouling agents are usually biocides which are released from the surface of the paint over a period of time at a concentration lethal to marine organisms at the hull surface. The anti-fouling paint fails only when the concentration of biocide available at the paint surface falls below the lethal concentration, and with modern paints up to two years of useful life is expected.

30

An extremely widely used biocide, particularly in marine anti-foulants, is tributyl tin (TBT). However, there is a growing concern about the environmental effects caused by using such organic tin biocides at their present commercial levels as an anti-foulant active ingredient in coating compositions for aquatic (marine) applications. It has been  
5 shown that, due to the wide-spread use of tributyltin-type compounds in particular, at concentrations as high as 20% by weight in paints for ship hulls, the pollution of surrounding water due to leaching has reached such a level as to cause the degradation of mussel and shell organisms. This toxicity is clearly a problem with prior art antifoulants.

10 Furthermore, these polluting effects have been detected along the French-British coastline and a similar effect has been confirmed in U.S. and Far Eastern waters. Under the most recent regulatory restrictions, with limited exceptions, pleasure boats up to 25 meters long are no longer permitted to use anti-foulant paint containing high  
15 levels of tributyltin compounds.

There is clearly a desire to provide alternative antifoulants to TBT based compounds.

US-A-4297137 discloses that the effects of an anti-fouling composition can be  
20 lengthened by moderating the release of the anti-fouling constituents. This document discloses anti-fouling paints comprising at least one substance toxic to marine organism uniformly incorporated into a discontinuous solid matrix which is insoluble in sea water and is dispersed in the paint. The matrix is at least partially formed from at least one substance which becomes soluble in sea water under the action of enzymes  
25 liberated by marine organisms and/or by bacteria in contact with the paint. Thus, when a marine organism becomes associated with the painted surface, the toxic substance is released and the organism's growth is inhibited. Similar to prior art disclosures, the toxic substances envisaged by US-A-4297137 include the well known copper and tin based compounds, such as TBT. Clearly, even controlled release of such prior art  
30 compounds pollutes the environment, albeit at lower levels than uncontrolled release. Furthermore, as the toxic compounds are released from these prior art antifoulant

paints, the effective life of the paint as an antifoulant is reduced, since once all the toxic compound has been released, the coating will no longer function as an antifoulant. This is a problem of such prior art antifoulant formulations.

- 5 Anti-inflammatory agents are useful to reduce inflammation, modulate allergic reactions, alleviate symptoms of asthma, treat conditions such as inflammatory bowel disease (e.g. Crohn's disease), ulcerative colitis, rhinitis, rheumatoid arthritis, psoriasis, interstitial cystitis, and control toxic shocks, among other uses. Heparin is a prior art product known to possess a wide array of anti-inflammatory properties. Heparin, however, is also a known anti-coagulant, reducing the ability of the blood to clot. Clearly, the anti-coagulant properties of heparin may be problematic in its use as an anti-inflammatory agent.

The present invention seeks to overcome such difficulties.

- 15 Aspects of the present invention are set out in the claims and are described below.

#### **Summary of the Invention**

- 20 In a first aspect, the invention relates to a product capable of having one or more properties selected from: anti-fouling properties; anti-adhesive properties; anti-inflammatory properties; and wherein said product is obtainable from starfish.

- Typically, the product will be free of at least one component with which it is usually  
25 associated in its natural environment.

Preferably the product is substantially free of the components with which it is usually associated in its natural environment.

- 30 More preferably the product is isolated from the components with which it is usually associated in its natural environment.

The term product as used herein may refer to a molecule or a plurality of molecules. The product of the present invention may preferably be obtained from starfish as disclosed herein. Most preferably, the product of the present invention may be molecule(s) obtainable from the mucus secretions of starfish. Preferably the starfish is *Marthasterias glacialis*. The product will preferably be obtained from said mucus secretions by collecting said secretions, and removing the particulate matter by centrifugation. The product may then be advantageously purified by size exclusion chromatography, and even more preferably be further purified by ion exchange chromatography.

In a highly preferred embodiment, the product of the present invention may be a proteoglycan obtainable from the mucus secretions of *Marthasterias glacialis*.

- 15 A proteoglycan (PG) is any glycoprotein which comprises a polypeptide or protein core with one or more glycosaminoglycans (GAGs) bound to it. A glycosaminoglycan (GAG) is a polysaccharide which has a simple repeating disaccharide unit and can be highly charged due to the presence of sulphate and carboxyl groups.
- 20 In the present specification "foulants" referred to by the terms "anti-foul(s)", "anti-fouling", and "anti-foulants" include molecules or other substances, or may include organisms which may reside and/or grow on the surface to be treated with the present composition. Such organisms may include micro-organisms such as bacteria, fungi, protozoa, algae or other micro-organism. The organism may be a marine organism.
- 25 Said marine organism may be a barnacle or limpet or any other organism capable of adhering to a substrate or surface.

The surface may be the surface of an organism, such as a mammal. The surface may be endothelial, for example vascular endothelium, or may be epithelial, for example bladder epithelium. The surface may be the surface of a prosthetic, or of an artificial implant. 'Fouling' describes the adhesion of said substances or organisms to said

surface(s).

Accordingly, anti-fouling properties will be any characteristics or abilities of a product to inhibit, reduce, reverse, prevent or otherwise interfere with, discourage or slow down the process of fouling.

Adhesion or adherence is used to describe the association, attachment, sticking, binding or bonding of a substance or organism to a surface or substrate. Therefore, anti-adhesive properties will be any characteristics or abilities of a product to inhibit, reduce, reverse, prevent or otherwise interfere with, discourage or slow down the process of adhesion.

Inflammation is a complex phenomenon observed in many organisms in response to wounding, infection, allergic reaction, toxic shock or the presence of many kinds of pathogen, as well as many other factors. When the organism is an animal, this often involves the migration of white blood cells to the site of inflammation, and cell-cell contacts or adhesion. Anti-inflammatory properties describe any abilities of an anti-inflammatory agent to reduce the symptoms or causes of inflammation. Preferably, the products of the present invention when used as anti-inflammatory agents may inhibit, reduce, reverse, prevent or otherwise interfere with, discourage or slow down the process of inflammation. More preferably, an anti-inflammatory property of a product according to the invention may refer to its ability to interfere with the adhesion of bacterial cells to mammalian cells, most preferably an anti-inflammatory property of a product according to the invention may refer to its ability to interfere with the adhesion of white blood cells such as leukocytes or neutrophils to endothelial cells such as vascular endothelium, or with epithelial cells such as bladder epithelium. However, it is to be understood that the anti-inflammatory properties are not necessarily limited to such activities.

Preferably, the product of the invention is obtainable from *Marthasterias glacialis*, more preferably said product is obtainable from the mucus secretions of *Marthasterias glacialis*.

5 Preferably, the product is a proteoglycan, or an active component thereof.

The term 'an active component' of the product of the present invention may refer to a purified fraction of the product. For example, if the product of the invention comprises a plurality of molecular species, an active component thereof may be a plurality of molecular species which is selected from the original mixture, or may be a single molecular species selected from said mixture. In other words, the active component may be a subset of the molecules which the product comprises. Furthermore, an 'active component' of the product as used herein may refer to a molecular sub-species of the original mixture. For example, if the product of the invention is a proteoglycan, an active component thereof may comprise the glycan chain(s) or part thereof, or may comprise the polypeptide entity or part thereof, or any combination of the two.

Thus, in one aspect, the product may be a glycan, or an active component thereof. Preferably, the glycan of the product is capable of displaying gas chromatography peaks as shown in Figure 4, and as described in Table 3 (See Example 2).

The active component will preferably retain the activity of the product according to the invention. Preferred methods for assessing the activities of the product according to the present invention are described herein and will be known to those skilled in the art. Preferably, the activities of the product of the present invention are anti-fouling properties, anti-adhesive properties, and anti-inflammatory properties. Preferably, the product of the present invention, or an active component thereof, will possess at least one of said properties, more preferably will possess at least two of said properties, and most preferably will possess at least all three of these properties.

Preferably, the product or active component thereof has one or more characteristics selected from:

- i) a molecular weight of about 1,100 kDa as measured by 3% polyacrylamide gel electrophoresis
  - 5 ii) capable of displaying a Fourier transform infra-red spectrum similar to that shown in Figure 3, with the peaks indicated in Table 2
  - iii) capable of displaying a NMR proton spectrum similar to that shown in Figure 1
  - iv) sensitivity to the action of chondroitinase ABC I
  - v) sensitivity to the action of *N*-glycanase
  - 10 vi) resistance to the action of chondroitinases ACI and B
  - vii) resistance to the action of proteinase K
  - viii) resistance to the action of papain
  - ix) sensitivity to the action of neuraminidase
- 15 The terms sensitivity/resistance as used herein are qualitative. Sensitivity/resistance may be estimated by exposing the product to the particular enzyme(s) of interest, and comparing the chromatographic profiles of product exposed to the enzyme(s) with the chromatographic profiles of product which has not been exposed to the enzyme(s). If the chromatographic profiles differ, then it would be inferred that the product exhibited
- 20 sensitivity to the enzyme(s), whereas if the profiles were essentially the same, the product would be judged to be resistant to the enzyme(s). Further, sensitivity or resistance to a particular enzyme may be estimated in a similar manner by comparing the SDS-PAGE profiles of samples of product which had either been treated with enzyme, or had not been treated with enzyme. As explained above, a difference
- 25 between the profiles would be taken to indicate sensitivity to the enzyme(s), whereas if the profiles were essentially the same, the product would be judged to be insensitive or resistant to the enzyme(s). Further explanation of these assessments can be found in Examples 1 and 2.
- 30 Preferably said product has two or more of said characteristics, more preferably three or more of said characteristics, more preferably four or more of said characteristics,

even more preferably five or more of said characteristics, even more preferably six or more of said characteristics, yet more preferably seven or more of said characteristics, yet more preferably eight or more of said characteristics, and most preferably all of said characteristics.

5

Preferred methods for assessing said characteristics may be found in the examples.

In a further embodiment, the product of the invention preferably will not have significant anti-coagulant properties. As used herein, significant anti-coagulant properties are anti-coagulant properties comparable to those of heparin. Any product having anti-coagulant properties less than those of heparin is considered not to have significant anti-coagulant properties.

Coagulation refers to the thickening or clotting of blood and assays for determination of coagulation or anti-coagulant properties are known in the art such as the Acuclot and Heptest diagnostic tests, or for example see Thompson and Harker 1983 (*Manual of Hemostasis and Thrombosis* Davis Company, Philadelphia).

In a further embodiment, the invention relates to a method for the preparation of a product according to the present invention, said method comprising

- i) collecting mucus from *Marthasterias glacialis*,
- ii) removing particulate material by centrifugation
- iii) subjecting the supernatent to column chromatography
- iv) eluting the material from the chromatography column of (c), and
- v) optionally dialysing said eluted material against distilled water.

The present invention also encompasses a method for preparing a pharmaceutical composition, said method comprising admixing a product of the present invention with a pharmaceutically acceptable carrier, diluent or excipient.

30

The present invention also encompasses a method for preparing an antifouling composition, said method comprising admixing a product of the present invention with a suitable vehicle, solvent, carrier, diluent or excipient.

- 5 The present invention also encompasses a method for preparing an anti-adhesive composition, said method comprising admixing a product of the present invention with a suitable vehicle, solvent, carrier, diluent or excipient.

- 10 The invention also relates to an antibody raised against the product according to the invention, or an antibody which has immunoreactivity with such a product, or a compound capable of having immunoreactivity with such an antibody.

#### **Detailed Description of the Invention**

- 15 Typically, the product of the present invention is present in a composition.

The compositions of the present invention may be formulated as coatings, lacquers, stains, enamels and the like, hereinafter referred to generically as "coating(s)".

- 20 Thus, in one aspect the present invention provides a coating consisting of a composition as defined above.

The coating may include a liquid vehicle (solvent) for dissolving or suspending the composition.

- 25 The liquid vehicle may be selected from any liquid which does not interfere with the activities of any essential components of the composition. In particular, the liquid vehicle should not interfere with the activity of the anti-foulant compound. Suitable liquid vehicles are disclosed in (for example) US-A-5071479 and include water and organic solvents including aliphatic hydrocarbons, aromatic hydrocarbons, such as xylene, toluene, mixtures of aliphatic and aromatic hydrocarbons having boiling points

between 100 and 320°C, preferably between 150 and 230°C; high aromatic petroleum distillates, e.g., solvent naphtha, distilled tar oil and mixtures thereof; alcohols such as butanol, octanol and glycols; vegetable and mineral oils; ketones such as acetone; petroleum fractions such as mineral spirits and kerosene, chlorinated hydrocarbons.

5 glycol esters, glycol ester ethers, derivatives and mixtures thereof.

The liquid vehicle may contain at least one polar solvent, such as water, in admixture with an oily or oil-like low-volatility organic solvent, such as the mixture of aromatic and aliphatic solvents found in white spirits, also commonly called mineral spirits.

10

The vehicle may typically contain at least one of a diluent, an emulsifier, a wetting agent, a dispersing agent or other surface active agent. Examples of suitable emulsifiers are disclosed in US-A-5071479 and include nonylphenol-ethylene oxide ethers, polyoxyethylene sorbitol esters or polyoxyethylene sorbitan esters of fatty acids, derivatives and mixtures thereof.

15

Any suitable surface coating material may be incorporated in the composition and/or coating of the present invention. Examples of trade-recognized coating materials are polyvinyl chloride resins in a solvent based system, chlorinated rubbers in a solvent based system, acrylic resins and methacrylate resins in solvent based or aqueous systems, vinyl chloride-vinyl acetate copolymer systems as aqueous dispersions or solvent based systems, butadiene copolymers such as butadiene-styrene rubbers, butadiene-acrylonitrile rubbers, and butadiene-styrene-acrylonitrile rubbers, drying oils such as linseed oil, alkyd resins, asphalt, epoxy resins, urethane resins, polyester resins, phenolic resins, derivatives and mixtures thereof.

20

The composition and/or coating of the present invention may contain pigments selected from inorganic pigments, such as titanium dioxide, ferric oxide, silica, talc, or china clay, organic pigments such as carbon black or dyes, derivatives or mixtures thereof.

25

The composition or coating of the present invention may contain materials such as rosin to provide controlled release of the anti-foulant compound, rosin being to a very slight extent soluble in sea water.

- 5 The composition and/or coating of the present invention may contain plasticizers, rheology characteristic modifiers, other conventional ingredients or mixtures thereof.

- 10 The composition or coating of the present invention, particularly the coating, may further comprise an adjuvant conventionally employed in compositions used for protecting materials exposed to an aquatic environment. These adjuvants may be selected from additional fungicides, auxiliary solvents, processing additives such as defoamers, fixatives, plasticizers, UV-stabilizers or stability enhancers, water soluble or water insoluble dyes, colour pigments, siccatives, corrosion inhibitors, thickeners or anti-settlement agents such as carboxymethyl cellulose, polyacrylic acid or polymethacrylic acid; anti-skimming agents, derivatives or mixtures thereof.
- 15

The additional fungicide(s) used in the composition and/or coating of the present invention will preferably be soluble in the liquid vehicle.

- 20 Thus, in one aspect the present invention provides antifoulant (such as a marine anti-foulant) comprising the product of the present invention.

Preferably, the anti-foulant is self-polishable.

- 25 The composition of the present invention can be provided as a ready-for-use product or as a concentrate. The ready-for-use product may be in the form of an aqueous solution, aqueous dispersion, oil solution, oil dispersion, emulsion, or an aerosol preparation. The concentrate may be used, for example, as an additive for coating, or may be diluted prior to use with additional solvents or suspending agents.

30

An aerosol preparation according to the invention may be obtained by methods known

to one skilled in the art by incorporating the composition of the present invention comprising or dissolved or suspended in, a suitable solvent, in a volatile liquid suitable for use as a propellant, for example the mixture of chlorine and fluorine derivatives of methane and ethane commercially available under the trademark "Freon", or  
5 compressed air, or other suitable propellant.

As discussed in US-A-5071479 the composition or coating of the present invention may include additional ingredients known to be useful in preservatives or coatings. Such ingredients include fixatives such as carboxymethylcellulose, polyvinyl alcohol,  
10 paraffin, co-solvents, such as ethylglycol acetate and methoxypropyl acetate, plasticizers such as benzoic acid esters and phthalates, e.g., dibutyl phthalate, dioctyl phthalate and didodecyl phthalate, derivatives and mixtures thereof. Optionally dyes, color pigments, corrosion inhibitors, chemical stabilizers or siccatives (dryers) such as cobalt octate and cobalt naphthenate, may also be included depending on specific  
15 applications.

The composition or coating of the present invention can be applied by any of the techniques known in the art including brushing, spraying, roll coating, dipping or combinations thereof.

20 Compositions of the present invention can be prepared simply by mixing the various ingredients at a temperature at which they are not adversely affected. Equipment and methods conventionally employed in the manufacture of coating or similar compositions may be advantageously employed.

25 According to a further aspect, the invention relates to an antibody which is capable of reacting with the proteoglycan (PG) product described herein.

30 The antibody may be used to isolate further quantities of the product of the present invention and/or to detect the presence of the product of the present invention.

The term "antibody", as used herein with reference to the present invention, refers to a complete antibody or an antibody fragment or an antibody component, as well as any combination thereof, capable of binding to the selected target - namely the product of the present invention, or an active component thereof.

5

The term "antibody" refers to both conventionally produced antisera and monoclonal and engineered antibody molecules.

Antibody fragments and components include Fv, ScFv, dsFv, Fab, F(ab), Fab', F(ab)2, 10 F(ab')2, Facb, monoclonal and polyclonal antibodies, engineered antibodies including chimeric, CDR-grafted antibodies, and artificially selected antibodies produced using phage display or alternative techniques. Small fragments, such Fv and ScFv, possess advantageous properties for analytical applications.

15 Preferably the antibody is linked to a detectable moiety.

Any suitable detectable moiety can be used. The moiety can be directly detectable - such as a radiolabelled moiety, a moiety comprising a dye that is capable of producing a visually detectable signal (which need not necessarily be detectable by means of the naked eye) or 20 a luminescent moiety. The moiety can be indirectly detectable - such as an enzyme moiety that is capable of acting on a substrate that is itself capable of generating a detectable signal or a moiety that is itself recognised by a labelled antibody.

The term "linked" includes direct attachment - such as through a direct bond, e.g. an 25 ionic bond or a covalent bond.

Polyclonal antibodies (antisera) may be prepared by conventional means which comprise inoculating a host animal, for example a mouse, rat or a rabbit, with a polypeptide of the invention or peptide fragment thereof and recovering immune serum.

30

Techniques for the preparation of antibodies are discussed in, for example, Kohler and Milstein, (1975) Nature 256:495-497; US 4,376,110; Harlow and Lane, Antibodies: a Laboratory Manual, (1988) Cold Spring Harbor, incorporated herein by reference. Techniques for the preparation of recombinant antibody molecules is also described in the above references and also in, for example, EP 0623679; EP 0368684 and EP 0436597, which are incorporated herein by reference.

By way of example, cell culture supernatants may be screened for the desired antibodies, preferably by immunofluorescent staining of the product according to the invention by immunoblotting, by an enzyme immunoassay, e.g. a sandwich assay or a dot-assay, or a radioimmunoassay.

For isolation of the antibodies, the immunoglobulins in the culture supernatants or in the ascitic fluid may be concentrated, e.g. by precipitation with ammonium sulphate, dialysis against hygroscopic material such as polyethylene glycol, filtration through selective membranes, or the like. If necessary and/or desired, the antibodies are purified by the customary chromatography methods, for example gel filtration, ion-exchange chromatography, chromatography over DEAE-cellulose and/or (immuno) affinity chromatography, e.g. affinity chromatography with the product of the present invention.

As mentioned above, the present invention also covers pharmaceutical compositions comprising the products of the invention. In this regard, and in particular for human therapy, even though the products of the present invention (including their pharmaceutically acceptable salts and pharmaceutically acceptable solvates) can be administered alone, they will generally be administered in admixture with a pharmaceutical carrier, excipient or diluent selected with regard to the intended route of administration and standard pharmaceutical practice.

By way of example, in the pharmaceutical compositions of the present invention, the products of the present invention may be admixed with any suitable binder(s), lubricant(s), suspending agent(s), coating agent(s), solubilising agent(s).

- 5 In general, a therapeutically effective daily oral or intravenous dose of the products of the invention is likely to range from 0.00001 to 500 mg/kg body weight of the subject to be treated, preferably 0.1 to 20 mg/kg. The products of the present invention may also be administered by intravenous infusion, at a dose which is likely to range from 0.00001-1000 mg/kg/hr.
- 10 Tablets or capsules of the products may be administered singly or two or more at a time, as appropriate. It is also possible to administer the products in sustained release formulations.
- 15 Typically, the physician will determine the actual dosage which will be most suitable for an individual patient and it will vary with the age, weight and response of the particular patient. The above dosages are exemplary of the average case. There can, of course, be individual instances where higher or lower dosage ranges are merited, and such are within the scope of this invention.
- 20 Alternatively, the products of the invention can be administered by inhalation or in the form of a suppository or pessary, or they may be applied topically in the form of a lotion, solution, cream, ointment, foam (e.g. similar to Predfoam®), or dusting powder. An alternative means of transdermal administration is by use of a skin patch. For example, they can be incorporated into a cream consisting of an aqueous emulsion of polyethylene glycols or liquid paraffin. They can also be incorporated, at a concentration of between 1 and 10% by weight, into an ointment consisting of a white wax or white soft paraffin base together with such stabilisers and preservatives as may be required.
- 25
- 30

For some applications, preferably the compositions are administered orally in the form of tablets containing excipients such as starch or lactose, or in capsules or ovules either alone or in admixture with excipients, or in the form of elixirs, solutions or suspensions containing flavouring or colouring agents.

- 5       The compositions (as well as the products alone) can also be injected parenterally, for example intracavernosally, intravenously, intramuscularly or subcutaneously. The compositions (as well as the products alone) may also be injected via the intrathecal/epidural routes. In this case, the compositions will comprise a suitable  
 10      carrier or diluent.

- 15      For parenteral administration, the compositions are best used in the form of a sterile aqueous solution which may contain other substances, for example enough salts or monosaccharides to make the solution isotonic with blood. Said solutions may be used as an instillation (for example bladder, as in interstitial cystitis), or a rectal or vaginal wash, the salt/monosaccharide composition being adjusted accordingly to suitable levels for such applications.

- 20      For buccal or sublingual administration the compositions may be administered in the form of tablets or lozenges which can be formulated in a conventional manner.

- 25      For oral, parenteral, buccal and sublingual administration to subjects (such as patients), the daily dosage level of the products of the present invention may typically be from 0.001 to 500 mg (in single or divided doses). Thus, and by way of example, tablets or capsules may contain from 0.001 to 500 mg of active product for administration singly, or two or more at a time, as appropriate. As indicated above, the physician will determine the actual dosage which will be most suitable for an individual patient and it will vary with the age, weight and response of the particular patient. It is to be noted that whilst the above-mentioned dosages are exemplary of the average case there can, of course, be individual instances where higher or lower dosage ranges are merited and such dose ranges are within the scope of this invention.  
 30

Thus the invention provides a pharmaceutical composition comprising a product of the present invention, together with a pharmaceutically acceptable diluent, excipient or carrier.

5

The invention further provides a product of the present invention, or a pharmaceutical composition containing same, for use as a medicament. The medicament may be for human usage or veterinary usage.

- 10 The present invention will now be described by way of example, in which reference is made to:

Figure 1 which shows a spectrum;

- 15 Figure 2 which shows a spectrum;

Figure 3 which shows a spectrum; and

Figure 4 which shows a spectrum.

20

**Brief description of the figures:**

In slightly more detail:

- 25 Figure 1 shows an NMR proton spectrum of the proteoglycan product prepared as described herein.

Figure 2 shows FTIR spectra recorded from mucus glycoproteins.

**Figure 3** shows FTIR spectra recorded from mucus glycoproteins, with annotated peaks.

**Figure 4** shows a GC trace recorded from proteoglycan subjected to methanolysis,  
5 with annotated peaks.

**EXAMPLE 1: COLLECTION AND PURIFICATION OF PROTEOGLYCAN  
(PG) PRODUCT**

10 The starfish *Marthasterias glacialis* and *Porania pulvillus*, and the brittlestar *Ophiocomina nigra* are kept in through-flow seawater aquaria at a density of 20-40 animals per 500 litre tank.

Mucus is collected from *M. glacialis* and *O. nigra* in response to physical stress.

15 Animals are first blotted with paper towels to remove excess seawater. Large amounts of mucus are secreted in response to agitation with a glass rod. Mucus is also collected from *M. glacialis* and *P. pulvillus* using suction. The mucus is aspirated from the dorsal surface of both species using a fine glass Pasteur pipette connected to a reservoir under suction.

20 Mucus collected by aspiration is a viscous acidic liquid. Stress mucus is less viscous and of lower pH (see table 1 below).

25 Size exclusion chromatography of mucus from all three species of echinoderm produces characteristic chromatograms.

Mucus samples are clarified by centrifugation at 500g for 10 min and applied to a (95cm long x 26mm diameter) column of Sepharose CL-6B (Pharmacia), which has previously been calibrated with high molecular weight standards. The absorbance of 30 the eluant at 280 nm is monitored and fractions are collected. Protein content of samples is assayed using Coomassie® Plus reagent (Pierce). Glycosaminoglycan

(GAG) content of fractions are assayed using the dye dimethyl methylene blue with heparin and chondroitin sulphate C as standards, and as described below.

A major peak of GAG and protein elutes in the void volume, indicating the presence of  
5 a high molecular weight glycoprotein in all three mucus samples. The fractions from this peak are pooled for subsequent analysis / purification (see below).

The bulk of the glycoproteins present in the mucus elute in the void volume of the column. These glycoproteins are then dialysed against distilled H<sub>2</sub>O and freeze-dried.  
10 Reconstituted samples and PG standards are applied to a Q-Sepharose high performance column (Pharmacia) and eluted with a rising concentration of NaCl (0 to 1M in 0.02M Tris-HCl buffer pH 8.0 over 10 minutes). The absorbance of the eluant at 280 nm is monitored and fractions are collected. Pooled fractions are dialysed against distilled H<sub>2</sub>O and freeze dried for long term storage at -20 °C.

15

Table 1. Composition of echinoderm mucus

	Volume (ml per animal)	pH	[protein] μg. ml <sup>-1</sup>	[GAG] μg. ml <sup>-1</sup>
<i>M. glacialis</i> -stress	5	4.5	59	32
<i>M. glacialis</i> -normal	0.5	5	156	255
<i>P. pulvillus</i>	1	5	238	205
<i>O. nigra</i> -stress	0.5	4	57.4	18

Mucus secretions from *M. glacialis* contained an average of 210 μg/ml (+ or - 78μg/ml S.E.M. - Standard Error of the Mean; n=5) of proteoglycans.

20

## EXAMPLE 2: CHARACTERISATION OF PG PRODUCT

### Molecular Weight

The Molecular Weight (MW) of the product is estimated by Sodium Dodecyl Sulphate

- 5 - Polyacrylamide Gel Electrophoresis (SDS-PAGE) using 3% polyacrylamide gels.

The MW is found to be approximately 1,100 kDa.

### Susceptibility to proteinases

Purified or semi-purified mucus glycoproteins are digested with proteinases (proteinase

- 10 K and papain), neuraminidase, N-glycanase, and chondroitinases ACI, B, and ABC, and heparinases. The digests are analysed by ion-exchange chromatography using the Q-sepharose high performance column as described in Example 1. A change in the chromatographic profile indicates susceptibility to digestion.

- 15 The proteoglycan content of fractions collected from columns is estimated using the dimethylmethylen blue assay for sulphated GAGs as described in (Farndale *et al.*, 1986: Biochem.Biophys.Acta vol 883, pp171-177). Heparin and chondroitin sulphate C are used as standards.

- 20 The protein content of fractions collected from columns is measured using Coomassie® Plus reagent (Pierce) according to the manufacturer's instructions.

The uronic acid content of proteoglycans is determined after acid hydrolysis (6.0 M HCL at 100°C for 6h) by the modified carbazole reaction as described in (Bitter *et al.*,

- 25 1962: Analytical Biochemistry vol 4, pp330-334), and the hexosamine content was determined by the Elson-Morgan reaction according to (Rondic *et al.*, 1955: Biochem. J. vol 61, pp586-589).

The uronic acid content of the proteoglycan product is approximately 19 µg uronic acid per 1 mg PG. Similarly, the hexosamine content of the proteoglycan product is approximately 19 µg hexosamine per 1 mg PG. Radiolabelling indicates approximately 100 mol sulphate per mol GAG.

5

#### NMR characterisation of PG product.

It is possible to obtain 1D 500 MHz  $^1\text{H}$  NMR spectra of the mucus glycoproteins. It is possible to record a TOCSY spectrum at 70°C from which spin systems can be deduced.

10

Samples are dissolved in 99.8%  $\text{D}_2\text{O}$  and transferred to 5mm NMR tubes. Proton and carbon NMR spectra are recorded using a Varian Unity 500 NMR spectrometer, at temperatures of 45 or 70 degrees C.

15

Figure 1 shows an NMR proton spectrum of the proteoglycan product prepared as described herein. This spectrum is recorded at 500MHz, in  $\text{D}_2\text{O}$  solution, at 70 °C. The numbers on the scale are parts per million (ppm) and are expressed relative to an internal standard compound, 3-(trimethylsilyl)propionic acid  $d_4$  sodium salt. The peak from residual water is labelled, as are the reference peak, and the peaks in the spectrum most characteristic of the carbohydrate part of the molecule, the anomeric signals (ie those from the anomeric hydrogen, or H1, of each monosaccharide residue).

20

#### Fourier transform infra-red characterisation of PG product.

25

Freeze-dried samples of mucus glycoprotein are analysed by means of FTIR spectroscopy, using a Nicolet Magna-IR 860 spectrometer E.S.P. equipped with a liquid-nitrogen cooled mercury-cadmium-telluride (MCT) detector. Spectra are obtained by utilising a single-bounce ATR (attenuated-total reflection) zinc-selenide prism, over the range 680 - 4000  $\text{cm}^{-1}$ .

- FTIR spectra are recorded from mucus glycoproteins, and are shown in Figures 2 and 3, and peak assignment annotations are presented in table 2. The sulphate groups gave strong signals: SO<sub>2</sub> -stretch 1335 -1175 cm<sup>-1</sup>, SO-R 1100-770 cm<sup>-1</sup>, S=O stretch 1100 cm<sup>-1</sup>. There are features at about 1650 cm<sup>-1</sup> which appear to arise from the N-acetyl group on the hexosamine sugar. There is a prominent feature at about 1050 which, in combination with the apparent absence of a significant peak at about 1200, indicates that the *M. glacialis* glycoprotein is mainly 4-sulphated, without significant 6-sulphate and probably little, if any, N-sulphate.
- 5
- 10 The glycoproteins elute as a major peak, and one or more minor peaks, from a Q-Sepharose ion exchange column. The composition of *M. glacialis* glycoprotein indicates that it is relatively pure after size exclusion chromatography. The other products produce more complex chromatograms, indicating the presence of several species within the size-exclusion preparations.
- 15
- 15 The ion-exchange purified mucus glycoproteins from *M. glacialis* and *P. pulvillus* are resistant to digestion by proteinases K and papain. They are also resistant to neuraminidase and chondroitinases ACI and B, and heparinases, but a change in chromatographic profile indicates that they are sensitive to digestion by *N*-glycanase and chondroitinase ABC I. These results indicate that these glycoproteins are proteoglycans. Labelling with <sup>35</sup>SO<sub>4</sub> suggests that the proteoglycan is sulphated.
- 20

**Table 2: FTIR peak assignment information (see Figure 3)**

Vibrations Specific to Mucus proteoglycans	
1062	Sugar rings, many vibrations
1230	Sulphate, S=O stretch
1372	-OH bend
1463	CH <sub>2</sub> scissor
1519	N-acetyl group, N-H band
1644	N-acetyl group, C=O stretch
1734	Carbonyl group
Vibrations not specific to proteoglycans	
2334	CO <sub>2</sub>
2360	CO <sub>2</sub>
2849	C-H
2922	C-H
3269	-OH

**Notes to Table 2:**

- Reproducible spectra may be obtained using different machines.
- Spectra indicate that the disaccharide repeating unit of GAG chains is probably N-acetyl galactosamine-6-sulphate and glucuronic acid.

**Analysis of monosaccharides from *M. glacialis* proteoglycan**

Proteoglycan product from *M. glacialis* is subjected to methanolysis, and the resulting products are analysed by gas chromatography (GC).

5

GC data are presented in Table 3, and in Figure 4 below.

**Table 3: Gas Chromatography (GC) analysis of monosaccharides from *M. glacialis* proteoglycan after methanolysis.**

Retention Time	Monosaccharide
10.27	Unidentified pentose
10.76	Unidentified pentose
14.88	Mannose
14.99	Galactose
15.72	Galactose
16.34	Galactose
16.88	Glucose
17.23	Glucose
21.01	N-acetyl glucosamine

10

**EXAMPLE 3: ANTIFOULANT PROPERTIES**

**(i) Inhibition of bacterial adhesion (anti-adhesive properties)**

15 Bacterial adhesion to cells such as bladder epithelial cells can be important in the inflammatory response. Inhibition of such adhesion may indicate anti-inflammatory properties.

**Adhesion assay with vital staining.**

In order to assess the anti-foulant/anti-adhesive properties of the mucus proteoglycans in regulating bacterial adhesion, a flow-chamber as described in (Usami *et al.*, 1993:

5 Biomed Eng vol 21 pp77-83) is used. The chamber is adapted in order to accommodate a removable microscope slide within a window in its base. A PTFE gasket separates the lid and base of the chamber. The assembly is designed to fit onto the stage of an inverted fluorescent microscope in order to carry out real time video microscopy studies.

10

*Pseudomonas fluorescens* (NCIMB, Pf 1079) cells are grown overnight at room temperature in Anderson's marine medium. Cultures are washed 3 times with filtered seawater (FSW) and then stained with the vital fluorescent dye SYTO9® (Molecular probes). Stained bacteria are diluted to 50 ml and incubated for 1 h in seawater alone

15

or in seawater containing 1mg/ml of mucus or control PG.

Fluorescent-labelled bacteria are pumped at a flow rate of 0.025 ml per second, which produces a linear range of shear of approximately 43 - 0 dyn. cm<sup>-2</sup>. New glass microscope slides are fitted in the chamber for each experiment prior to pumping.

20

Short sequences of the passage of bacteria through the chamber are recorded, in regions of low and high shear, by video microscopy. On completion of recording the chamber is flushed with filtered sea water and photographs are taken of adhered bacteria.

25

These studies indicate that mucus glycoproteins from *M. glacialis* but not from *P. pulvillus* or *O. nigra* are effective in inhibiting bacterial adhesion.

**Adhesion assay with radioactive labelling.**

In order to quantify the effect of mucus glycoproteins on bacterial adhesion a static assay is used to measure the adhesion of radiolabeled bacteria to model surfaces.

- 5      *Pseudomonas fluorescens* are grown overnight at room temperature in Anderson's marine medium supplemented with 2.5  $\mu$ Ci/ml  $^3$ H-methyl thymidine (Amersham Life Science Ltd., Amersham, U.K.). Cells from such cultures are pelleted by centrifugation at 250g for 300s. Labelled bacteria are washed three times with filtered sea water (FSW) (0.2 $\mu$ m filtration) and re-suspended in FSW alone or FSW containing  
 10     0 - 1 mg/ml of mucus glycoprotein. They are then incubated for 3 h in 96-well tissue culture plates (Corning Costar Ltd., High Wycombe, U.K.). The suspension is discarded, and plates are rinsed 3 times with FSW. Adhered bacteria are lysed with 200  $\mu$ l of 0.2 M NaOH, 1 % SDS for 10 min and then neutralised with 200  $\mu$ l of 0.2 M HCl. Radioactive label is quantified by scintillation counting after the addition of  
 15     approx. 5ml of Optiphase scintillation fluid (Zinsser Analytical Ltd., Maidenhead, U.K.).

Control PG and mucus glycoproteins are used to coat the wells of tissue culture plastic 96-well plates in order to measure the effect of immobilised PG on bacterial adhesion.

- 20     Wells of 96-well plates are coated with poly-L-lysine (0.01 % -  $1 \times 10^{-6}$  %,) and glycoproteins ( $1 \times 10^{-5}$  ml at a concentration of 1 mg/ml) prior to adhesion assays. 200 $\mu$ l of solution is incubated in each appropriate well for 1 hour after which the solution is discarded and the plates are dried at 60°C for 1 hour. Adhesion of radiolabelled *P. fluorescens* is measured as described above.

- 25     Glycoproteins from *P. pulvillus* increase bacterial adhesion to tissue culture plastic in a dose-dependent manner. Mammalian heparin does not alter bacterial adhesion to tissue culture plastic. Dermatan sulphate increases bacterial adhesion to tissue culture plastic.  
 30

Studies of static adhesion using radiolabelled bacteria indicate that adhesion to tissue culture plastic is inhibited by 64%-68% by *M. glacialis* PG (34 µg/ml), but is enhanced by 131 % by *P. pulvillus* PG. Adhesion is not significantly affected by glycoproteins from *O. nigra*. Heparin has no effect on adhesion. Dermatan sulphate (100µg/ml) increases adhesion by 58%.

In order to measure adhesion of bacteria to the starfish cuticle, radiolabelled bacteria are incubated for 3 h with tube feet from *M. glacialis*. The tube feet are removed with scissors from a single specimen and rinsed with FSW. The contribution of surface groups to bacterial antifouling is assessed by digestion with specific enzymes. Tube feet are incubated with chondroitinase ABC, chondroitinase ACI, chondroitinase B or sulfatase enzymes. Controls are treated with digestion buffer only. Following digestion tube feet are rinsed with seawater, weighed and placed in wells of a 96-well plate. Radiolabeled bacteria (prepared as above) are than added and their adhesion is measured as described above. All treatments are performed in triplicate and adhesion results are normalised to the weight of each tube foot.

There is measurable adhesion of labelled bacteria to tube feet. This is increased by 20% by digesting tube feet with chondroitinase ABC. Treatment with chondroitinases ACI or B, or sulfatase, has no effect on bacterial adhesion. This indicates that a chondroitinase-sensitive surface PG contributes to the antifouling properties of the product. Chondroitinase ABC treatment reduces the anti-adhesive effect of the product.

Further studies of bacterial adhesion suggest that *M. glacialis* PG inhibits adhesion by causing bacteria to stick together in clumps. These clumps are visible flowing through the chamber and settle in areas of low flow, but are washed away in areas of fast flow.

#### (ii) Inhibition of adhesion of neutrophils to HUVECs

30

Adhesion of leukocytes or neutrophils to endothelial cells (such as vascular endothelial cells) can be an important event in the inflammatory response. Therefore, a product which inhibits this adhesion may possess anti-inflammatory properties.

**5 Cellular adhesion assay.**

In order to assess the effect of mucus proteoglycans (PGs) on cellular adhesion, an assay as described in (Kyan-Aung *et al.*, 1991: J. Immunol. vol. 146, pp521-528) is used to measure the interaction between leukocytes and human vascular endothelial

10 cells (HUVECs).

Polymorphonuclear leukocytes (> 95% neutrophils) are isolated from citrated (not heparinised) human venous blood using Percoll gradients (density dependent centrifugation).

15

Cryo-preserved HUVECs (TCS Ltd. U.K.) are cultured in endothelial cell basal medium (MCDB 131) with appropriate supplements. Cells are passaged by trypsinisation and grown to confluence in the central wells of flat-bottomed 96-well plates. For adhesion assays, cells are used at the fifth passage.

20

Monolayers of HUVECs are stimulated for six hours with IL-1 $\beta$  (10U/ml), LPS (2.5mg/ml) or TNF- $\alpha$  (125U/ml) in the absence and presence of mucus glycoproteins diluted in saline, or heparin and poly-glutamic acid controls. Following stimulation, monolayers are washed to remove stimuli and inhibitors, before the addition of  $2 \times 10^5$  radiolabelled leukocytes to each well. Following a 30 minute incubation at 37 °C non-adherent cells are removed by gentle aspiration and washing. The adherent cells in each well are lysed with 1% Nonidet-P40, placed in scintillation vials and counted on a  $\gamma$ -counter.

The adhesion of leukocytes stimulated with the peptide fMLP to unstimulated HUVECs is also measured. Plates are incubated for a further 30 minutes at 37 °C, and are then treated as described above.

- 5 Initial cell adhesion studies are carried out with unpurified samples of mucus glycoprotein from *M. glacialis*. These proved to be highly cytotoxic, causing cell lysis within 5 minutes. Partially purified mucus glycoproteins are found not to be cytotoxic, and inhibit neutrophil adhesion in a dose dependent manner (0.0001 - 1 mg/ml range).
- 10 Adhesion of leukocytes to endothelial cells is inhibited by proteoglycans (PGs) from *M. glacialis*.

Adhesion of radiolabelled human leukocytes to endothelial cells is inhibited by 68% by proteoglycans from *M. glacialis*.

15

#### **EXAMPLE 4: RAISING OF ANTIBODIES AGAINST PRODUCT**

##### **Raising of polyclonal antibodies against proteoglycan (PG) product**

- 20 Three aliquots of 150µg, 250µg and 250µg of purified proteoglycan (PG) antigen are emulsified with equal volumes of Freuds adjuvant and injected intramuscularly into a rabbit over a period of three weeks at intervals of 1, 7 and 21 days. Blood was collected two weeks after the final injection and incubated at room temperature for 1h and at 4°C overnight to allow for clotting. The serum is centrifuged twice at 5000g for 25 10min before an equal volume of glycerol is added to the serum. Sodium azide is added to a final concentration of 0.02% (w/v). This antiserum is stored at -20°C.

**EXAMPLE 5: LACK OF ANTICOAGULANT EFFECT COMPARED TO  
HEPARIN**

5      *M. glacialis* PG produced according to the invention does not have a significant anti-coagulant effect when compared with heparin in a coagulation assay.

Inhibition of coagulation or clotting (ie anti-coagulant properties) are estimated by measuring the activated partial thromboplastin time (APTT) according to Thompson and Harker 1983 (*Manual of Hemostasis and Thrombosis* Davis Company, 10 Philadelphia).

Treatment	APTT clotting time (n= 5)
control clotting time	39.2 ± 1.4 s
0.1mg per ml mucus PG	39.9 ± 1.6s
0.1 µg per ml mucus PG	39.1 ± 1.5 s
50 heparin units per ml	> 600s
0.05 heparin units per ml	43.8 s

Thus, proteoglycan preparations according to the invention have potent anti-adhesive properties in both mammalian cell and bacterial cell adhesion (see above) without 15 anticoagulant activity.

All publications mentioned in the above specification are herein incorporated by reference. Various modifications and variations of the described methods and system of the invention will be apparent to those skilled in the art without departing from the scope and spirit of the invention. Although the invention has been described in connection with specific preferred embodiments, it should be understood that the 20

invention as claimed should not be unduly limited to such specific embodiments. Indeed, various modifications of the described modes for carrying out the invention which are obvious to those skilled in chemistry or related fields are intended to be within the scope of the following claims.

CLAIMS

1. A product capable of having one or more properties selected from
  - a) anti-fouling properties,
  - b) anti-adhesive properties,
  - c) anti-inflammatory properties,wherein said product is obtainable from starfish.
2. A product according to claim 1 wherein the starfish is *Marthasterias glacialis*.
- 10 3. A product according to claim 2 wherein said product is obtainable from the tube feet of *Marthasterias glacialis*.
4. A product according to any of claims 1 to 3 wherein said product is obtainable from the mucus secretions of *Marthasterias glacialis*.
- 15 5. A product according to any of claims 1 to 4 wherein said product is a proteoglycan, or an active component thereof.
6. A product according to any previous claim, wherein said product has one or 20 more characteristics selected from
  - a) a molecular weight of about 1,100 kDa as measured by 3% polyacrylamide gel electrophoresis
  - b) capable of displaying a Fourier transform infra-red spectrum similar to that shown in Figure 3, with the peaks indicated in Table 2
  - 25 c) capable of displaying a NMR proton spectrum similar to that shown in Figure 1.
7. A product according to any previous claim, said product not having significant 30 anti-coagulant properties.

8. A method for the preparation of a product according to any previous claim, said method comprising
- a) collecting mucus from *Marthasterias glacialis*,
  - b) removing particulate material by centrifugation
  - c) subjecting the supernatent to column chromatography
  - d) eluting the product from the chromatography column of (c), and
  - e) optionally dialysing said eluted product against distilled water.
- 10 9. An antibody raised against a product according to any of claims 1 to 7, or a product obtainable by the method of claim 8.
10. An antifoulant comprising the product of any of claims 1 to 7, or a product obtainable by the method of claim 8.
- 15 11. A pharmaceutical comprising the product of any of claims 1 to 7, or a product obtainable by the method of claim 8.
12. An anti-adhesive comprising the product of any of claims 1 to 7, or a product obtainable by the method of claim 8.
- 20

ABSTRACTPRODUCT

The invention relates to a product capable of having one or more properties selected from; anti-fouling properties, anti-adhesive properties, anti-inflammatory properties,  
5 and wherein said product is obtainable from starfish.

Reference peak

Residual water

$^1\text{H}$  NMR spectrum at 500 MHz of  
proteoglycan from *M. glacialis*

Recorded in deuterium oxide solution at 70 °C

Chemical shifts in ppm relative to internal  
3-(trimethylsilyl)propionic acid-d<sub>4</sub> sodium salt  
at 0 ppm.

Anomeric signals from  
carbohydrate material  
in 4.4 - 5.5 ppm region

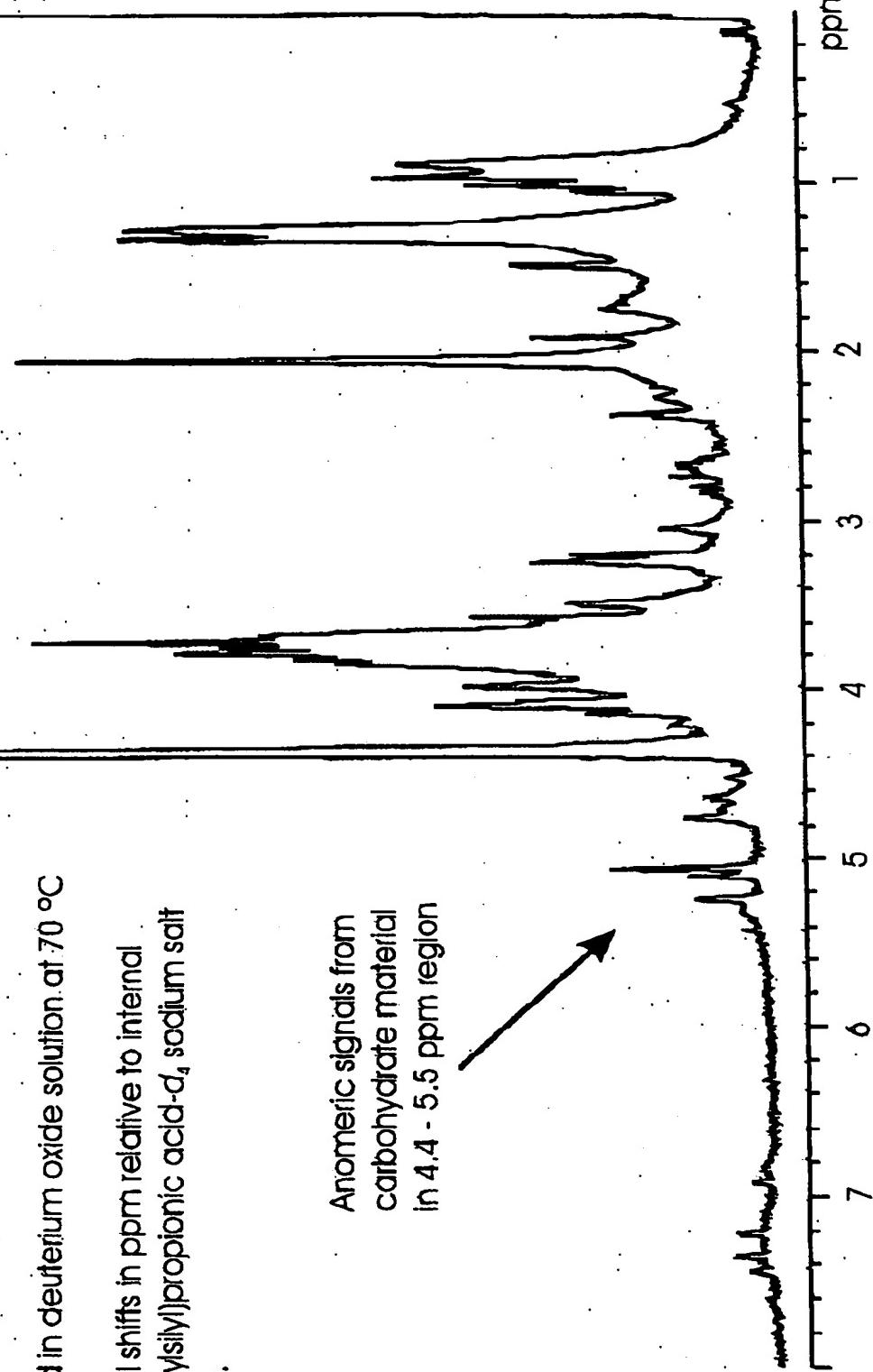


FIG. 1

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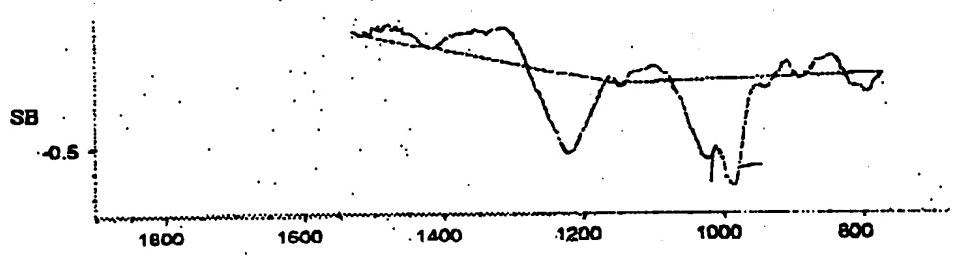


Figure 2: FTIR spectra recorded from mucus glycoproteins.

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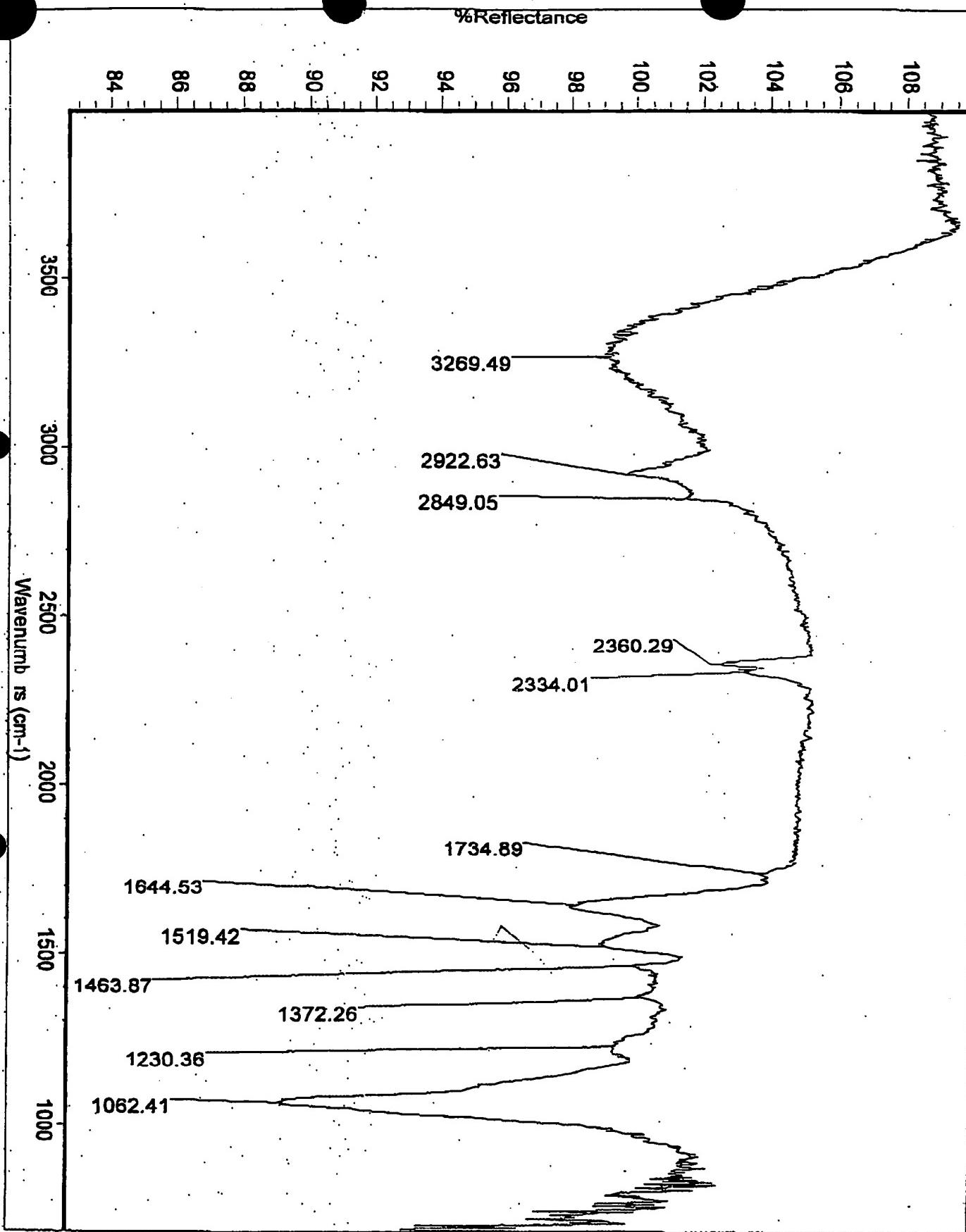
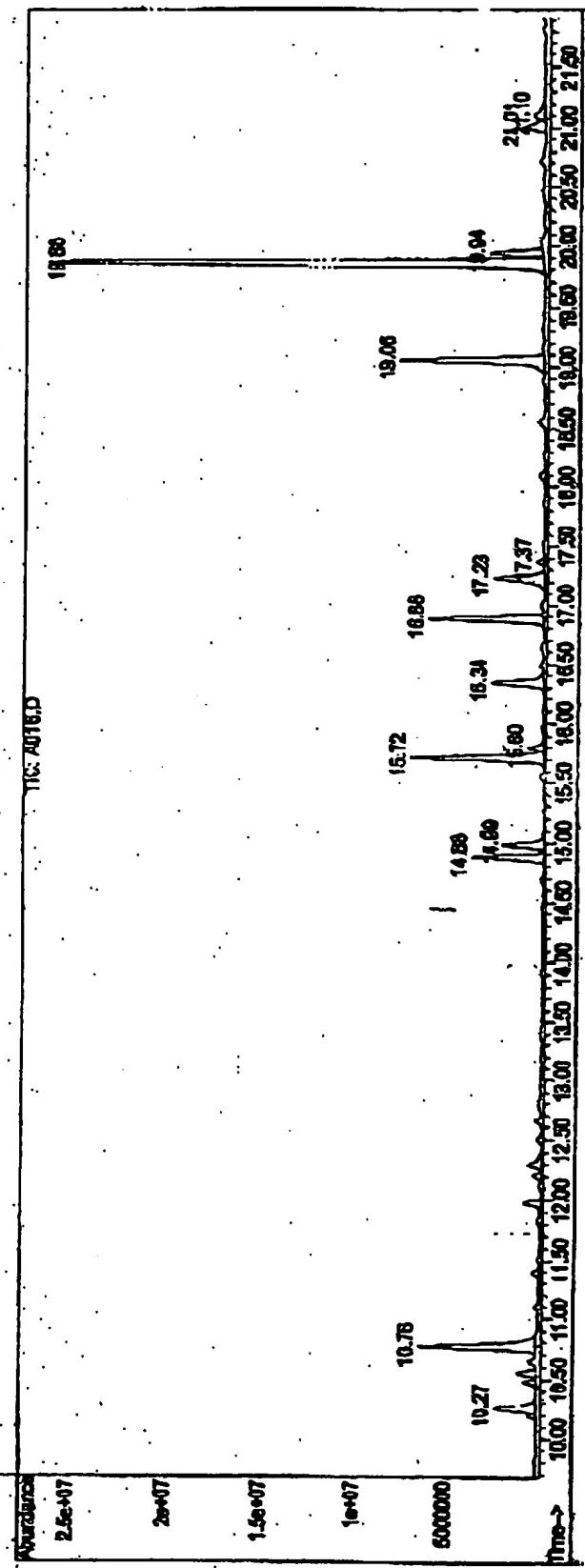


FIG. 3

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D. Young & Co.

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